Synthesis and Proinflammatory Effects of Peptidoglycan-Derived Neoglycopeptide Polymers

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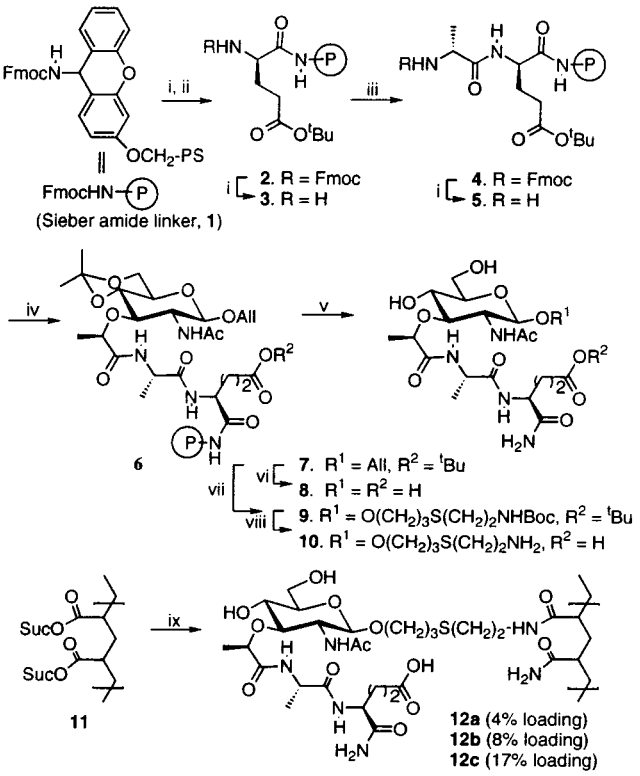
Lipopolysaccharides (LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA) comprise the three principal bacterial cell wall components implicated in inducing the clinical manifestations of septic shock.[1] These components exert their biological effects by stimulating the host's monocytes and macrophages to produce proinflammatory mediators, such as TNF-α, IL-1, and IL-6. These mediators in turn elicit a variety of proinflammatory responses in the host.

Recent studies indicate that the membrane-bound cluster differentiation antigen CD14 plays a key role in the signal transduction pathway leading to the production of the aforementioned proinflammatory mediators.[2] CD14 is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein that serves as a "pattern recognition" cell surface receptor for LPS, PGN, and LTA. This glycoprotein, which lacks transmembrane and cytoplasmic domains, is unable to directly transmit signals to the interior of the cell. That function is performed by Toll-like receptor 4 for LPS[3] and Toll-like receptor 2 for PGN and LTA.[4] While the precise mechanisms involved in the interactions between the various bacterial components, CD14 and the Toll-like receptors remain to be discovered, a complex of CD14 with a bacterial cell wall component has been implicated in the activation of a Toll-like receptor leading to the induction of cytokine gene expression.[5]

As part of a program designed to identify the structural requirements of bacterial cell wall components required for binding to CD14 and to induce the production of endogenous proinflammatory mediators, we report here the synthesis of a range of neoglycopeptide polymers (**12a**−**c**, Scheme 1) composed of a synthetic polyacrylamide backbone functionalized with pendent Muramyl dipeptide (MDP)[6] derivatives of differing densities. These synthetic polymers induced significantly greater production of TNF-α by a human monocyte cell line than did monomeric MDP. Furthermore, soluble PGN (sPGN) and a polymer having a high MDP-loading density (**12c**) exert their effects through CD14 and another as-yet unidentified receptor, whereas polymers with lower MDP-loading densities (**12a,b**) signal only through CD14.

Scheme 1[a]



[a] Reagents and conditions: i) 20% piperidine/DMF; ii) FmocGlu[t]-BuOH, PyBOP, HOBt, DIPEA, DMF; iii) FmocAlaOH, PyBOP, HOBt, DIPEA, DMF; iv) 2-*N*-Acetyl-1-*β*-*O*-allyl 4,6-*O*-isopropylidene muramic acid, PyBOP, HOBt, DIPEA, DMF; v) 3% TFA/CH_2Cl_2; vi) Pd/C, MeOH, Δ; then HCO_2H; vii) AIBN, $HS(CH_2)_2NHBoc$, dioxane, 75 °C; viii) 25% TFA/CH_2Cl_2; ix) compound **10**, Et_3N, DMF then NH_4OH.

The neoglycopeptide polymers **12a**−**c** were prepared by condensation of spacer-modified MDP **10** with preactivated poly-[*N*-acryloyloxy)succinimide (pNAS) (**11**) followed by quenching of the unreacted succinimate ester with aqueous ammonia.[7] By varying the ratio of **10** to activated esters in pNAS (**11**), a series of MDP-functionalized polymers was obtained with 4% (**12a**), 8% (**12b**), and 17% (**12c**) loading, respectively. The control compounds poly(acrylic acid) sodium salt and poylacrylamide were synthesized by treatment of pNAS (**11**) with sodium hydroxide or excess aqueous ammonia, respectively.

MDP (**8**) and spacer-modified MDP **10** were prepared by a polymer-supported approach (Scheme 1).[8] Thus, dipeptide **5** was synthesized on the hyper acid-sensitive Sieber amide resin using Fmoc-protected L-Ala-OH and D-Glu(*tert*-Bu)-OH as amino acids. PyBOP/HOBt was applied as the activating reagent to ensure efficient coupling using low equivalents of amino acids. In the last step of the assembly, **5** was coupled with 2-*N*-acetyl-1-*β*-*O*-allyl 4,6-*O*-isopropylidenemuramic acid to give **6**, which was cleaved from the polymeric support by treatment with 3% TFA in DCM. This reaction also resulted in removal of the isopropy-

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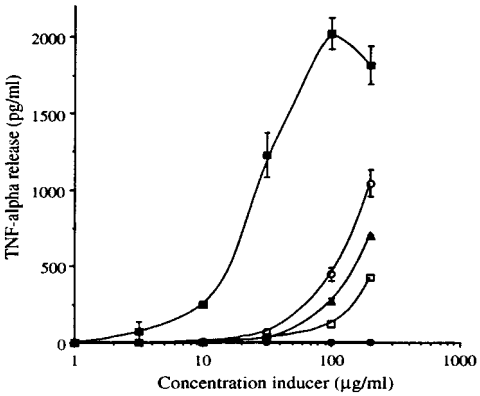

Figure 1. Induction of TNF-α release by Mono Mac 6 cells incubated with MDP (●), **12a** (□), **12b** (▲), **12c** (○), or sPGN (■). Mono Mac 6 cells (1×10^6/ml) were incubated with increasing concentrations of each stimulus as indicated for 6 h. The supernatants were harvested, and TNF-α release was determined by an ELISA assay. 1 and 10 ng/mL of LPS gave 423 ± 25 and 2128 ± 40 pg/mL TNF-α, respectively.

lidene group to give **7** in an overall yield of 91% based on initial resin loading. Compound **7** was converted into the spacer-containing derivative **10** by reaction with 2-[*tert*-butylcarbonyl-oxyamino]-1-ethane-thiol in the presence of the radical promoter AIBN in dioxane at 75 °C (→**9**)[9] followed removal of the Boc- and *t*-Bu-protecting groups.

The synthetic polymers **12a**−**c**, sPGN, and LPS all induced dose-dependent production of TNF-α by Mono Mac 6 cells, with the largest effect being caused by sPGN and LPS (Figure 1). Whereas monomeric MDP on its own caused very little production of TNF-α, higher densities of MDP conjugated to the polymer saw corresponding increases in TNF-α concentration. Neither the poly(acrylic acid) sodium salt nor the polyacrylamide induced any effect, indicating that the polymer backbone exerts no activity (data not shown). To exclude the possibility that TNF-α production arose from LPS contamination, the experiments were also preformed in the presence of polymyxin B, an antibiotic that avidly binds to the lipid A region of LPS, thereby preventing LPS-induced monokine production. TNF-α concentrations in supernatants of cells preincubated for 30 min with polymyxin B (1 μg/mL) before incubation with *E. coli O55:B5* LPS for 6 h fell to background levels, whereas preincubation with polymyxin B had no effect on TNF-α synthesis by cells incubated with **12a**−**c** or sPGN (see Supporting Information).

A number of synthetic polymers, functionalized with pendent saccharide moieties, have been shown to inhibit the binding of carbohydrate-binding proteins more avidly than their respective monomeric ligands.[10] This difference has been attributed to the now well-studied "*cluster effect*" wherein multivalent proteins display much higher affinities for multivalent ligands than their monomeric counterparts. That synthetic multivalent ligands might be exploited to trigger biological responses has, however, been more difficult to establish.[10c] We demonstrate here that synthetic

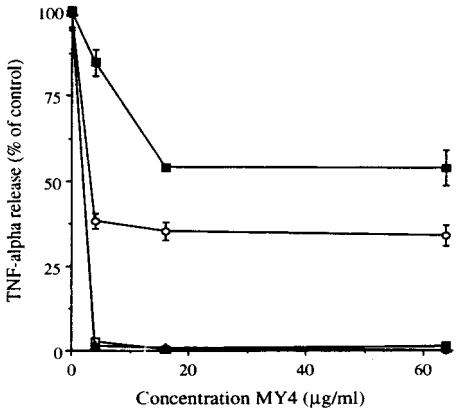

Figure 2. Inhibition of induced TNF-α release by anti-CD14 monoclonal antibody MY4. Mono Mac 6 cells (1×10^6/ml) were preincubated with anti-CD14 antibodies at increasing concentrations for 30 min at 4 °C. After 6 h of stimulation with 10 ng/mL LPS (●), 100 μg/mL **12a** (□),-100 μg/mL **12b** (▲), 100 μg/mL **12c** (○), or 10 μg/mL sPGN (■), the supernatants were harvested, and TNF-α release was determined by an ELISA assay.

polymers (**12a**−**c**) can activate a complex signal transduction pathway. The increased production of TNF-α compared with that of monomeric MDP may result from a "cluster effect". In fact, it has been suggested that soluble CD14 binds to MDP- or PGN-derivative monomers only when these are presented in a multimeric form.[11,12] Alternatively, it could be that the polymeric ligands assist receptor oligomerization, which may be a prerequisite for signal transduction.[13]

To determine whether activation of the Mono Mac 6 cells is mediated by CD14, modulation of TNF-α production by neutralizing anti-CD14 monoclonal antibodies MEM-18 and MY4 was studied. Both of these antibodies completely inhibited the effects of LPS and polymers **12a** and **12b** (Figure 2 for MEM-18, see Supporting Information). In contrast, the effects of sPGN and polymer **12c** were only partially inhibited. These unexpected observations indicate that LPS and polymers **12a** and **12b** stimulate TNF-α production uniquely through a CD14-dependent pathway, whereas polymer **12c** and sPGN can signal through an additional unidentified receptor.

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Supporting Information Available: Experimental procedures for the synthesis of compounds, dose response curve of LPS induced TNF-α production by Mono Mac 6 cells, effect of polymyxin B on TNF-α production, inhibition of induced TNF-α release by anti-CD14 monoclonal antibody MEM-18, effect of mouse immunoglobulin G2b and G1 as control for MY4 and MEM-18 antibodies, respectively (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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